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APPLICATION NUMBER: *60/503,689*
FILING DATE: *September 17, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/30066*

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[ ] Additional inventors are being named on the _____ separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

CYANIDE SENSING COMPOUNDS AND USES THEREOF

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 20 — [ ] CD(s), Number

[ ] Drawing(s) *Number of Sheets* 10 — [ ] Other (specify)

[ ] Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE Marianne Fuierer

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DATE 9/17/03

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DOCKET NO.: 4115-185 PRV

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Approved for use through 04/30/2003. OMB 0651-0032
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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

[X] Applicant Claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	NA
Filing Date	9/17/03
First Named Inventor	GEDDES, ET AL.
Examiner Name	
Art Unit	
Attorney Docket No.	4115-185 PRV

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1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
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1254	1,450	2254	725	Extension for reply within fourth month	
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1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR § 1.129(a))	
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Name (Print/Type)	MARIANNE FUIERER	*Registration No. (Attorney/Agent)*	39983	*Telephone*	(919) 419-9350
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4115-185 PRV

UNITED STATES PROVISIONAL PATENT APPLICATION

OF

CHRIS D. GEDDES

RAMACHANDRAM BADUGU

AND

JOSEPH R. LAKOWICZ

FOR

CYANIDE SENSING COMPOUNDS AND USES THEREOF

Intellectual Property/Technology Law • P.O. Box 14329 • Research Triangle Park, N.C. 27709

Acronyms and Symbols:

BA – Boronic Acid,
BAF and **BAFs** – Boronic acid containing fluorophore/s
BAQ – N-(Benzyl)-6-aminoquinolinium
BAQBA – N-(2-Boronobenzyi)-6-aminoquinolinium bromide
K_{SV} – Stern-Volmer quenching constant
LD – Laser Diode
LED – Light Emitting Diode
TCSPC – Time-Correlated Single Photon Counting

1. Introduction

It is considered that cyanide is one of the most lethal poisons known [1-10]. The mechanism of toxicity for cyanide is by absorption. Absorption occurs through the lungs, GI track and skin. Cyanide is highly toxic because it inhibits oxygen utilization by cells, binding with ferric iron in cytochrome oxidase, blocking the oxidative process of cells. As such the tissues with the highest oxygen requirement (brain, heart and lungs) are the most affected by acute poisoning. However, cyanide poisoning is not common, but can occur from smoke inhalation from residential and industrial fires, and in people who work in the metal, mining, electroplating, jewelry manufacture and in the x-ray film recovery trades [1-14].

Numerous chemical and physiochemical methods for the detection and determination of cyanides, such as potentiometric, chromatographic, spectrophotmetric, flow injection and electrochemical analysis are used [1-12], but only potentiometric determination has been reported as offering continuous cyanide monitoring [13]. Blood cyanide levels for healthy persons have been reported as being ≈ 0.3 μM using a gas chromatography method [14], with lethal cyanide blood levels for fire victims in the cyanide concentration range 23-26 μM

[14,15], approximately 100 times higher than normal blood levels [14]. As such, there is a requirement for simple, cheap and fast technologies to both detect and determine cyanide levels up to lethal concentrations, < 20 μM.

It is widely accepted that ratiometric or lifetime based methods offer intrinsic advantages for both chemical and biomedical fluorescence sensing [16,17]. Fluorescence intensity measurements are typically unreliable away from the laboratory and can require frequent calibration/s due to a variety of chemical, optical or other instrumental related factors. Unfortunately, while fluorescent probes are known to be useful for many applications such as in fluorescence microscopy, fluorescence sensing and DNA technology, most sensing fluorophores only display changes in intensity in response to analytes and hence relatively few wavelength ratiometric probes are available today [16,17]. Some useful wavelength ratiometric probes are available for pH, Ca^{2+} and Mg^{2+} [18,19], but the probes for Na^{+} and K^{+} generally display small spectral shifts and negligible lifetime changes and are subsequently inadequate for quantitive sensing measurements [17].

In this paper we characterize a range of new boronic acid containing fluorophores, Figure 1 (BAFs), which show both spectral shifts and intensity changes for increasing cyanide concentrations, in a wavelength ratiometric manner, enabling cyanide to be sensed at physiological and lethal levels, < 20 μM. In addition, the wavelength changes upon cyanide complexation with the new BAQBA probes, Figure 2, affords for a colorimetric response towards cyanide, changing from green, in the *absence* of cyanide, to colorless by the

presence of as little as 10 μM CN^-. Given the importance of sensing cyanide in a simple and accurate manner [1-10], we believe that these new probes may find applications in field deployable bio-warfare / terrorism type devices as well as in clinical laboratories.

The origin of the cyanide response is due to the boronic acid group's ability to interact with bases such as CN^-, as shown in Figure 2, to form the tricyanide anion R-$B(CN^-)_3$, which is an electron donating group, the extent of which being dependent on the concentration of cyanide present. This in turn interacts with the electron deficient quaternary heterocyclic nitrogen center of the quinolinium backbone, affording for the wavelength shifts and intensity changes observed. Interestingly, by replacing the 6-amino group on the quinolinium backbone with less efficient electron donating groups, e.g. $-OCH_3$, $-CH_3$ etc, in essence making the nitrogen center relatively more electron deficient, then the emission bands at 450 and 546 nm are not observed in the presence of cyanide, eliminating the possibility of a ratiometric response. In contrast, a greater electron deficient nitrogen center, provides for a greater affinity for either monosaccharides or cyanide, due to charge stabilization of the complexed form [20]. The synthesis of these new probes and the effect of backbone substituents on the spectral properties of the BAQBA probes have been reported elsewhere [20].

2. Experimental

2.1 Materials

All chemicals were purchased from Sigma. The preparation of the *ortho*, *meta* and *para* forms of BAQBA and BAQ, Figure 1, has recently been reported by us [20].

2.2 Methods

All solution absorption measurements were performed in a 4*1*1 cm quartz cuvette (Starna), using a Cary 50 Spectrophotometer from Varian. Fluorescence spectra were similarly collected on a Varian Eclipse spectrofluorometer with solution optical densities less than 0.2 and λ_{ex} = 358 nm.

Stability (K_S - units μM^{-3} or $mol^{-3}\ dm^9$ for CN^- and mM^{-1} or $mol^{-1}\ dm^3$ for glucose and fructose) and Dissociation constants (K_D) were obtained by fitting the titration curves with aqueous sodium cyanide to the relation:

$$I = \frac{I_{min} + I_{max} Ks[cyanide]}{1 + Ks[cyanide]} \qquad (1)$$

where I_{min} and I_{max} are the initial (no cyanide) and final (plateau) fluorescence intensities of the titration curves, where K_D=($1/K_S$).

Time-resolved intensity decays were measured using reverse start-stop time-correlated single-photon counting (TCSPC) [16] with a Becker and Hickl gmbh 630 SPC PC card and an un-amplifed MCP-PMT. Vertically polarized excitation at ≈ 372 nm was obtained using a pulsed LED source (1 MHz repetition rate) and a dichroic sheet polarizer. The instrumental response function was ≈ 1.1 ns fwhm. The emission was collected at the magic angle (54.7^0), using a long pass filter (Edmund Scientific), which cut off wavelengths below 380 nm.

The use of a pulsed 372 nm LED provided for excitation near-to the isobestic point at 358 nm, Figure 3 Top. A 550 ± 10 nm interference filter was also used to study the long-wavelength emission band of the BAQBA probes.

The intensity decays were analyzed in terms of the multi-exponential model:

$$I(t) = \sum_i \alpha_i \exp(-t / \tau_i) \qquad (2)$$

where α_i are the amplitudes and τ_i the decay times, $\Sigma\ \alpha_i = 1.0$. The fractional contribution of each component to the steady-state intensity is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \qquad (3)$$

The mean lifetime of the excited state is given by:

$$\bar{\tau} = \sum_i f_i \tau_i \qquad (4)$$

and the amplitude-weighted lifetime is given by:

$$<\tau> = \sum_i \alpha_i \tau_i \qquad (5)$$

The values of α_i and τ_i were determined by non-linear least squares impulse reconvolution with a goodness-of-fit χ^2_R criterion [16].

3. Results and Discussion

Figure 3 shows the absorbance for both *o*-BAQBA and BAQ with increasing cyanide concentrations. As the cyanide concentration increases the absorption band at 388 nm decreases while the band at 340 nm increases. We can see significant changes in both bands as the cyanide concentration is increased, Figure 3 Top. As expected the absorption spectrum of BAQ is unchanged by the addition of cyanide, confirming our expectations that the boronic acid moiety of BAQBA binds cyanide as depicted in Figure 2, and that BAQ does not. To the best of our knowledge, the boronic acid group has not been reported to both bind and thus sense cyanide in this manner. All three BAQBA probes showed similar responses to cyanide. Subsequently, Figure 3 Bottom, shows the absorption wavelength ratiometric plots for all 3 BAQBA probes and BAQ based on the A_{340}/A_{388} nm bands. Interestingly, *m*-BAQBA shows a much stronger response, with a greater dynamic sensing range, as compared to the other two *ortho*- and *meta*- BAQBA probes.

The fluorescence emission of the BAQBA probes shows similar wavelength ratiometric behavior, Figure 4 Top, where λ_{ex} = 358 nm, i.e. at the isobestic point. As the cyanide concentration increases, we typically see a decrease in the 546 nm emission band and a subsequent increase in the 450 nm band, which is attributed to the emission of the cyanide bound complexed form. This ratiometric response can also be seen visually, Figure 5, where the vial on the left contains no cyanide and the vial on the right contains only 10 μM cyanide. This result strongly suggests the use of these BAQBA probes for cyanide

determination < 20 μM, which is important for physiological detection and safeguard [1-10]. In contrast, BAQ shows very little change in fluorescence intensity, with no ratiometric behavior observed.

We constructed the fluorescence emission wavelength ratiometric response, Figure 4 Bottom, all three BAQBA probes having a similar response to aqueous cyanide. By comparing Figures 3 and 4 bottom we can see that a greater change is observed for the ratiometric absorption measurements, reflecting the difference in extinction coefficients and quantum yields of the CN^- unbound and bound forms respectively. Using equation 1 and the data in Figure 4 Bottom, we were able to determine the cyanide binding constants for the *ortho*, *meta* and *para* boronic acid probes to be 0.12, 0.17 and 0.14 μM^{-3}, noting the units μM^{-3} or $mol^{-3}\ dm^{9}$.

We additionally measured the lifetime/s of the probes in the absence and presence of cyanide, using the well-known time-correlated single photon counting technique, TCSPC [16], to investigate the possibility of fluorescence lifetime ratiometric sensing, Figure 6 and Table I.

BAQ was found to be monoexponential in Millipore water with a lifetime of ≈ 2.49 ns, unperturbed by the addition of sodium cyanide, further strengthening our proposed cyanide binding mechanism as shown in Figure 2. This can be clearly seen in Figure 6 Top, where the addition of 20 μM NaCN does not perturb the intensity decay of BAQ.

We measured the lifetimes of the two emission bands of the BAQBA probes separately, using both a 380 nm long pass filter and a 550 nm ± 10

interference filter. Table I shows that the lifetime/s of the emission band at 550 nm is unaltered by aqueous NaCN, where both the mean and amplitude weighted lifetimes remain approximately constant. However, when we determine the lifetimes through a 380 long pass filter a short-lived component, < 400 ps, becomes evident at high CN^- concentrations, Table I, evident as a third component in the intensity decay. This can be seen visually in Figure 6 Bottom and is in contrast to that observed for BAQ. We subsequently assign this short-lived component to the lifetime of the CN^- bound complex form of the *o*-BAQBA. While this short lived species is measurable with our UV LED for excitation (fwhm ≈ 1.1 ns), it's ps lifetime prevents its *practical* use for ratiometric lifetime sensing [16,17]. Similar results were found for all 3 BAQBA probes, with a longer lifetime component additionally observed for *m*-BAQBA.

The affinity of boronic acid for diols is well known [21-23]. Subsequently we tested the response of the BAQBA probes towards glucose and fructose, and using equation 1 we were able to determine the binding constants for *o*- and *m*- to be 3.90 and 3.18 mM^{-1} for glucose, and 1.06 and 1.55 mM^{-1} for fructose (data not shown, and no data is available for *p*-BAQBA). Interestingly, the response for glucose was found to be higher than that for fructose, but all were significantly lower than determined for cyanide. While it is difficult to make direct comparisons because of the units for both are different, the relatively higher affinity for the cyanide anion suggests that monosaccharides, such as glucose and fructose, would not interfere in cyanide measurements. Subsequently, we measured the absorption and emission wavelength ratiometric response in the presence of a

constant background of 100 mM glucose or fructose, Figures 7 and 8 respectively. Interestingly, the presence of the sugars did not interfere with the cyanide measurements, similar results being determined for cyanide in both the absence (just in water) and presence of either 100 mM glucose or fructose. The relatively higher binding affinity for species by *m*-BAQBA was not surprising, given similar reports for other *meta*-positioned boronic acid groups on other fluorophores [24].

Finally, we tested the quenching of the BAQBA probes by aqueous chloride, which is known to quench some quinolinium fluorescence [25-27]. We determined the Stern-Volmer Constants, K_{SV} [25], for *o*-, *m*- and *p*- BAQBA all to be $\approx 1.0\ M^{-1}$, in essence displaying only a very weak quenching [25]. This was surprising as many quinolinium type fluorophores have much more notable responses towards chloride and are therefore used as chloride probes [16,25]. Subsequently, we tested both the absorption and emission wavelength ratiometric response of the BAQBA probes towards cyanide in the presence of a *physiological-like* cocktail of 50 mM glucose, 50 mM chloride and 5 mM fructose, Figures 9 and 10 respectively. Our results are most encouraging, and show that the response towards cyanide is maintained, and that these potential physiological interferences do not perturb the dynamic range for cyanide sensing, Figures 9 and 10 Bottom.

4.0 Conclusions

We have characterized the response of 3 new boronic acid containing fluorophores towards aqueous cyanide, and have shown that cyanide

concentrations less than 20 μM can readily be determined in both a ratiometric and colorimetric manner. By characterizing a similar probe, BAQ, which is identical except that it does not contain the boronic acid group, we can rationale that cyanide readily binds to the boronic acid moiety, in a similar manner to other anions [28].

The relatively higher binding constant for cyanide as compared to glucose and fructose, and the fact that chloride does not quench BAQBA florescence well, strongly suggests the use of these probes for physiological cyanide determination and safeguard. In addition, these new probes are readily water soluble, have high quantum yields [20], can be produced by a one-step synthesis [20] and are compatible with cheap UV LED and LD excitation sources or even ambient light for a colorimetric type measurement, i.e. Figure 5.

Acknowledgements

This work was supported by the National Center for Research Resources, RR-08119. Partial salary support to JRL from UMBI is also gratefully acknowledged.

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Figure L gends

Figure 1. – Molecular structure of *ortho*, *meta* and *para*-BAQBA probes and the control compound BAQ, which does not contain the boronic acid moiety.

Figure 2. – Equilibrium involved in the interaction between the boronic acid group and cyanide.

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Figure 9. – Absorption spectra of *o*-BAQBA with increasing cyanide concentrations, in the presence of 50 mM Glucose, 5 mM Fructose and 50 mM Chloride, **Top**, and the respective ratiometric plots (A_{340}/A_{388} nm bands) for *o*, *m* and *p*-BAQBA in the presence of the same *physiological-like* background cocktail with increasing cyanide concentrations, **Bottom**.

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bands) for *o*, *m* and *p*-BAQBA in the presence of the same *physiological-like* background cocktail, for increasing cyanide concentrations, **Bottom**.

Table 1 – Multiexponential Intensity decay of BAQ and *o*-BAQBA.

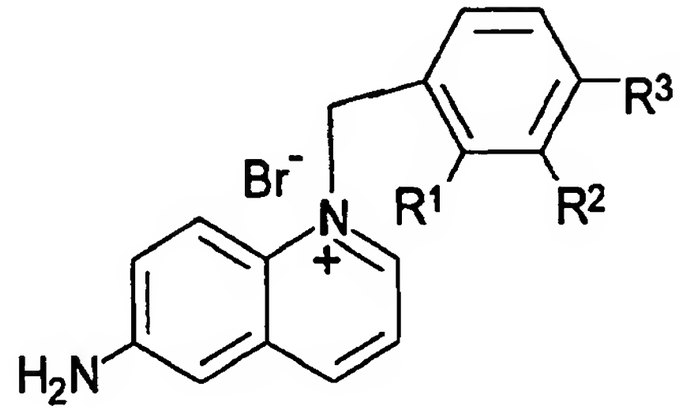
[Cyanide] μM	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	$\bar{\tau}$	$\langle\tau\rangle$	χ^2
BAQ									
0	2.48	1	-	-	-	-	2.48	2.48	1.10
2	2.48	1	-	-	-	-	2.48	2.48	1.02
4	2.49	1	-	-	-	-	2.49	2.49	1.19
6	2.49	1	-	-	-	-	2.49	2.49	1.32
10	2.49	1	-	-	-	-	2.49	2.49	1.18
16	2.49	1	-	-	-	-	2.49	2.49	1.28
20	2.47	1	-	-	-	-	2.47	2.47	0.89
***o*-BAQBA**									
(380 nm)[a]									
0	2.04	0.71	3.41	0.29	-	-	2.59	2.44	1.06
2	2.02	0.68	3.367	0.32	-	-	2.61	2.45	0.99
4	1.98	0.67	3.37	0.33	-	-	2.61	2.44	0.94
6	1.92	0.62	3.23	0.38	-	-	2.59	2.42	1.06
8[c]	1.55	0.41	2.98	0.59	-	-	2.60	2.39	1.53
10[c]	0.67	0.19	2.64	0.81	-	-	2.53	2.27	2.15
12.5	0.44	0.22	2.60	0.78	-	-	2.50	2.12	2.37
	0.21	0.17	2.07	0.63	3.99	0.20	2.76	2.14	1.08
15	0.38	0.28	2.61	0.72	-	-	2.49	1.98	2.18
	0.21	0.23	1.85	0.44	3.46	0.32	2.71	1.97	1.01
20	0.38	0.30	2.65	0.70	-	-	2.52	1.97	2.47
	0.19	0.24	1.69	0.39	3.36	0.37	2.72	1.95	1.12
(550 nm)[b]									
0	1.99	0.63	3.19	0.37	-	-	2.57	2.43	0.99
2	1.93	0.59	3.15	0.41	-	-	2.58	2.43	0.98
4	2.04	0.70	3.39	0.30	-	-	2.60	2.45	1.07
6	1.87	0.51	2.97	0.49	-	-	2.53	2.41	1.10
8	1.86	0.55	3.14	0.45	-	-	2.60	2.44	1.01
10	1.75	0.48	3.10	0.52	-	-	2.63	2.45	1.17
12.5	1.85	0.61	3.48	0.39	-	-	2.74	2.49	1.03
15	1.32	0.31	2.93	0.69	-	-	2.66	2.43	1.25
20	1.19	0.30	2.97	0.70	-	-	2.71	2.44	0.92

[a]380 nm long pass filter; [b]550 ± 10 nm interference filter; [c]No notable improvement in fit could be obtained using a 3-exp function. Similar values were also found for the *meta*- and *para*-BAQBA probes.

Claims

That which is claimed is:

1. A boronic acid containing fluorophore of the formula (I)



(I)

wherein R^1, R^2 and R^3 is hydrogen and $B(OH)_2$ with the proviso that the compound comprises one $B(OH)_2$ group.

2. The compound according to claim 1 wherein the $B(OH)_2$ group is in the ortho position.

3. The compound according to claim 1 wherein the $B(OH)_2$ group is in the meta position.

4. The compound according to claim 1 wherein the $B(OH)_2$ group is in the para position.

5. The compound according to claim 1 having an affinity for cyanide.

6. The compound according to claim 5 having binding sites for three CN anion.

7. A method of testing for low levels of cyanide, the method comprising
contacting a biological fluid potentially comprising a cyanide compound with a

compound according to claim 1 to determine binding of a cyanide compound thereto.

8. The method according to claim 7, wherein binding of the cyanide compound causes a change in fluorescence intensity.

9. The method according to claim 8, wherein as the number of binding cyanide anions increases, the fluorescence intensity increases.

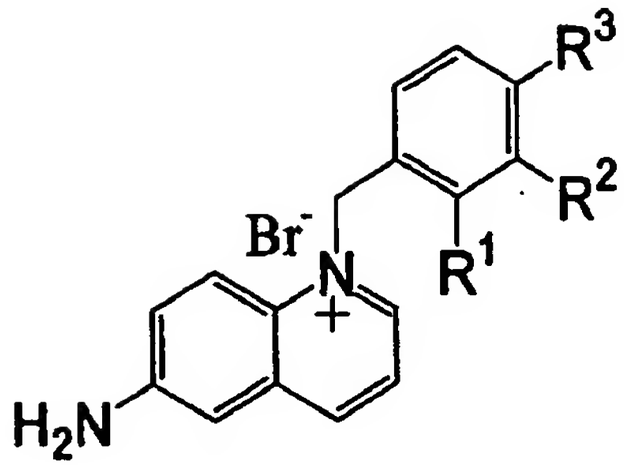
10. The method according to claim 7, wherein cyanide concentration can be sensed at levels less than 20 *u*M.

11. The method according to claim 8, wherein fluorescing is visible by use of a LED source.

12. The method according to claim 7, wherein the biological fluid is blood.

13. The method according to claim 7, wherein a colorimetric response is visible when cyanide binds to the compound of claim 1.

14. The method according to claim 7, wherein a wavelength shift occurs when cyanide binds to the compound of claim 1.



Probe	R^1	R^2	R^3
***o*-BAQBA**	$B(OH)_2$	H	H
***m*-BAQBA**	H	$B(OH)_2$	H
***p*-BAQBA**	H	H	$B(OH)_2$
BAQ	H	H	H

Figure 1. – Molecular structure of *ortho*, *meta* and *para*-BAQBA probes and the control compound BAQ, which does not contain the boronic acid moiety.

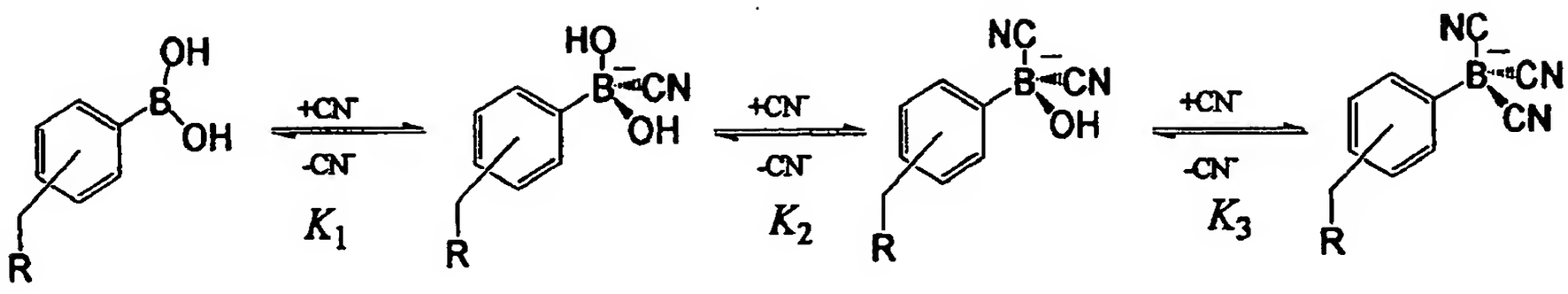


Figure 2. – Equilibrium involved in the interaction between the boronic acid group and cyanide.

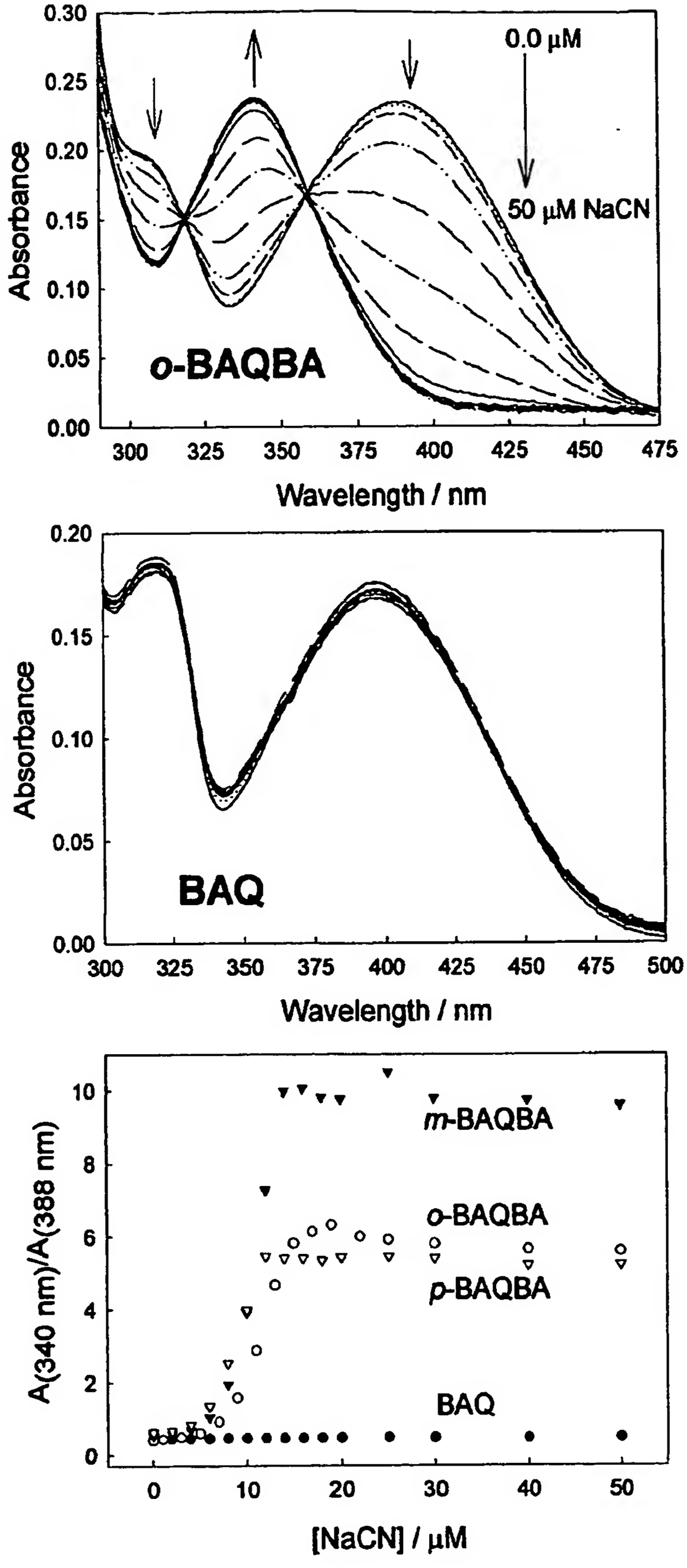
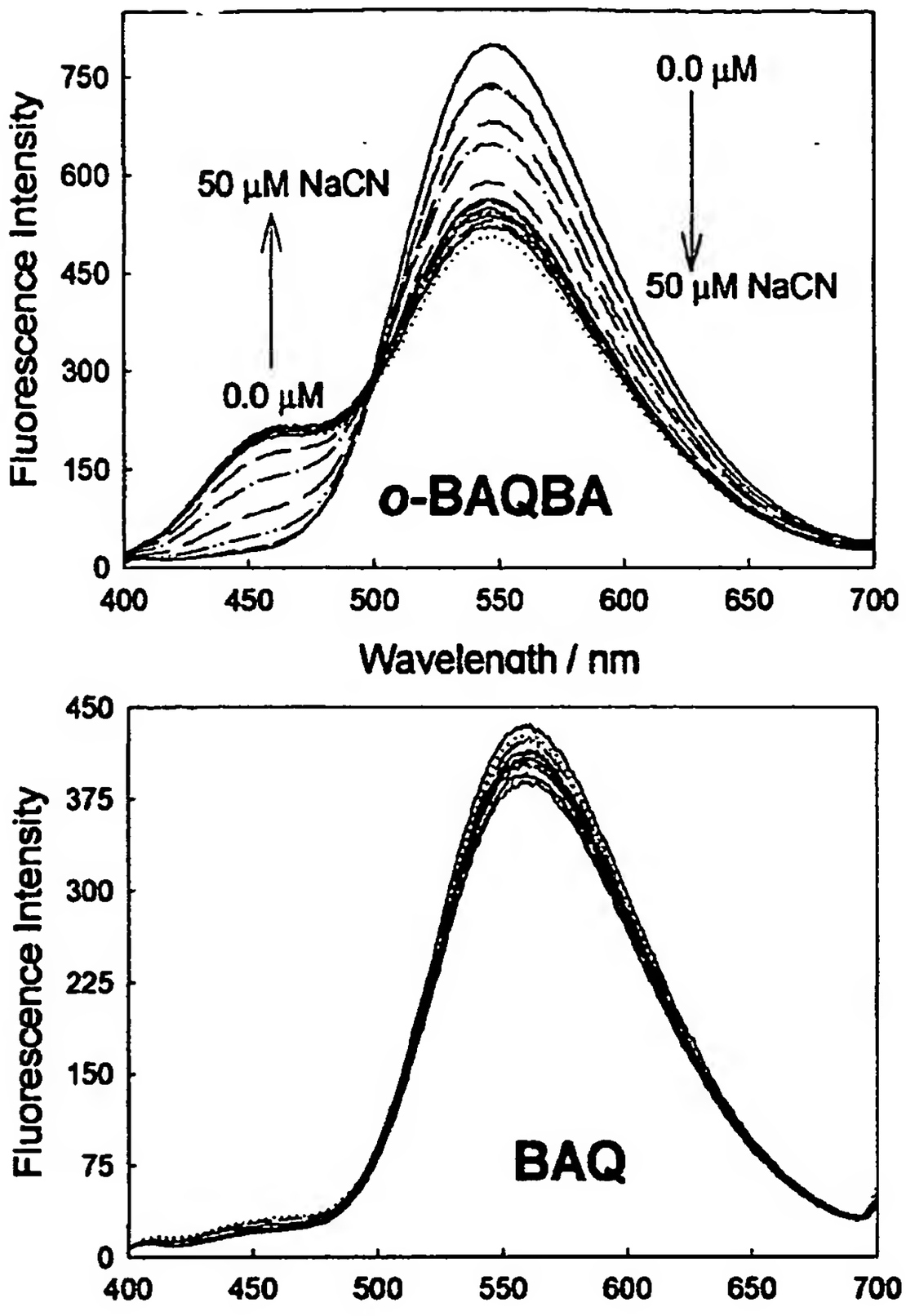


Figure 3. – Absorption spectrum of both *o*-BAQBA and BAQ with increasing cyanide concentration, **Top** and **Middle** respectively, and the respective wavelength ratiometric plots based on the A_{340}/A_{388} nm bands, **Bottom**.



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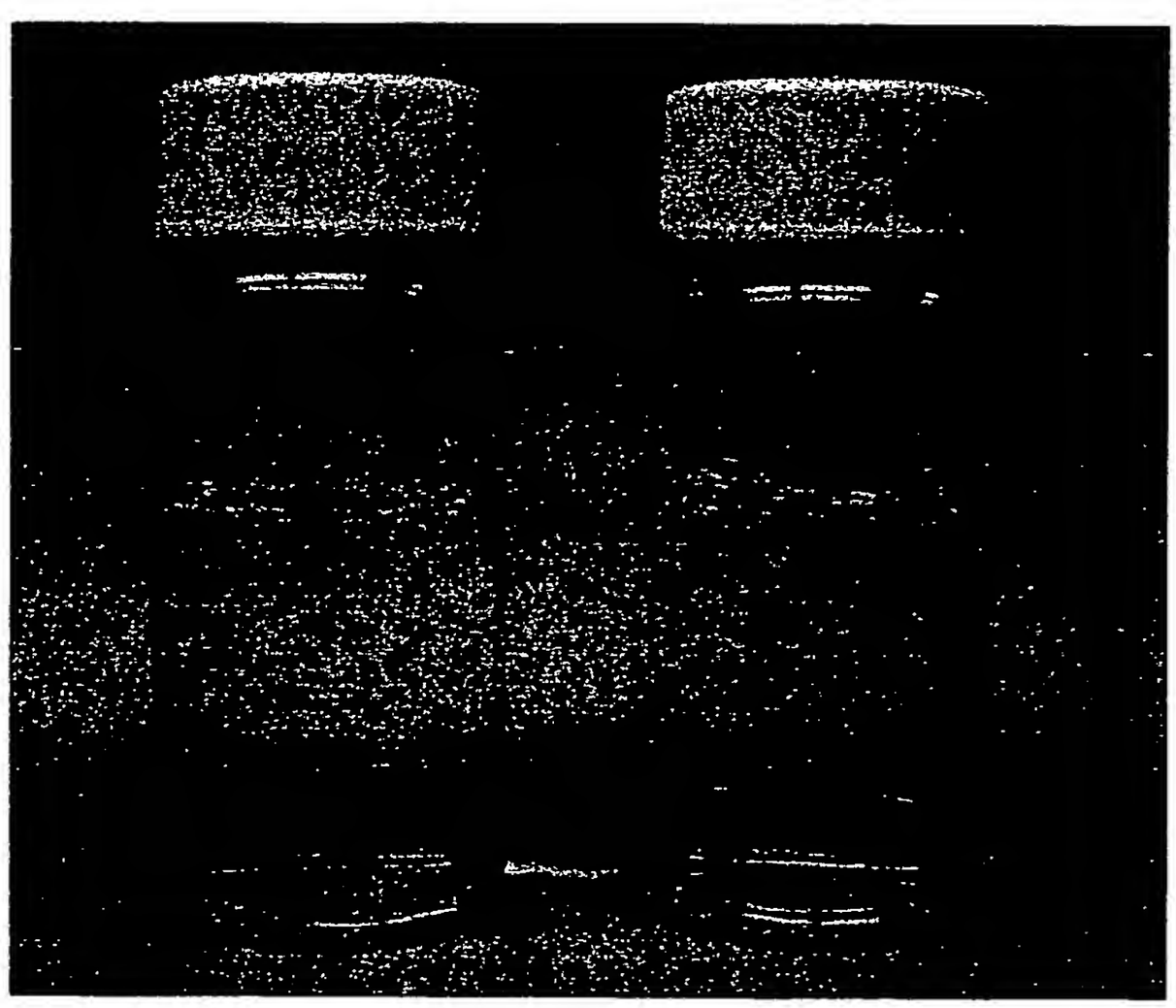

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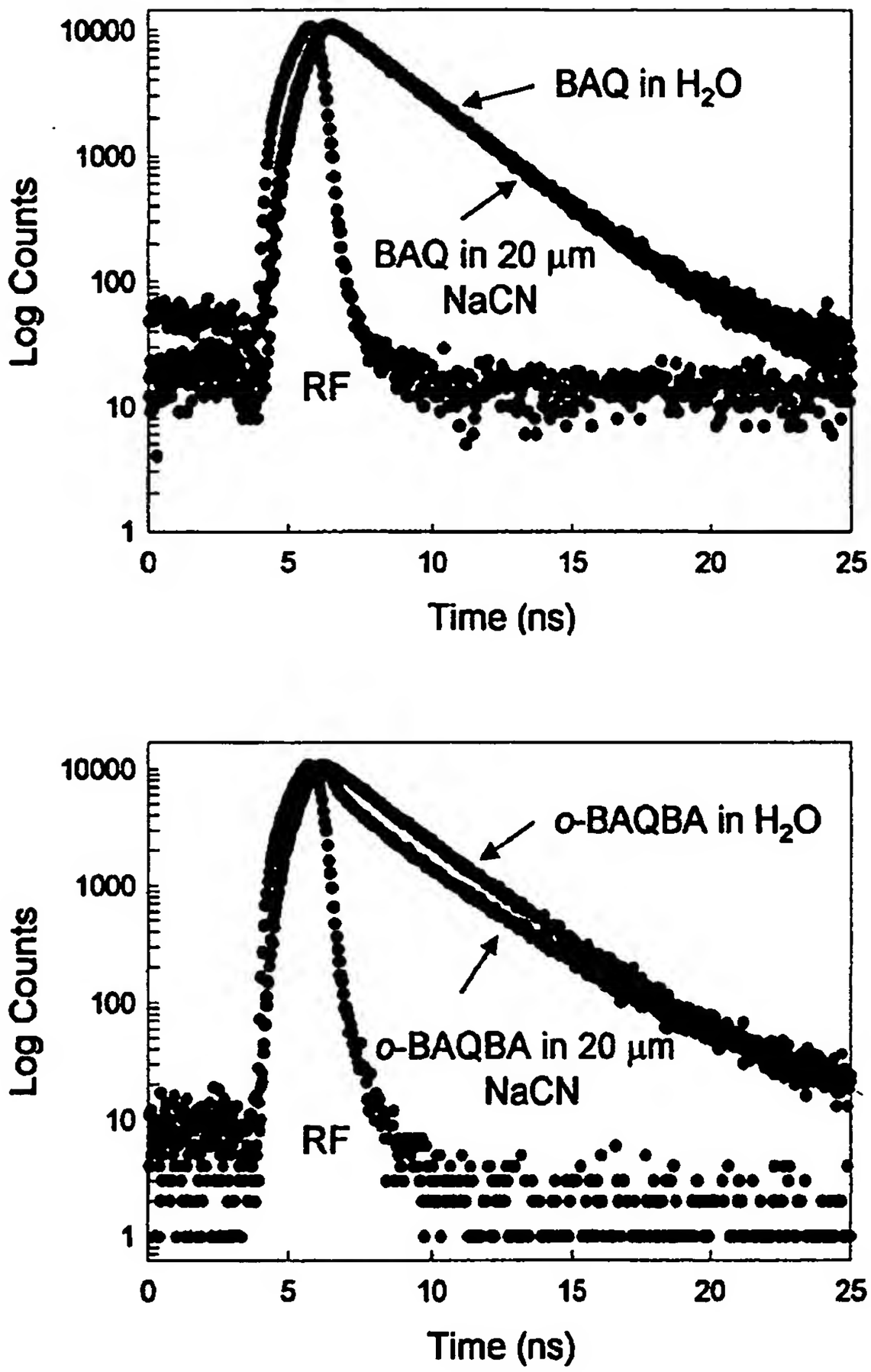

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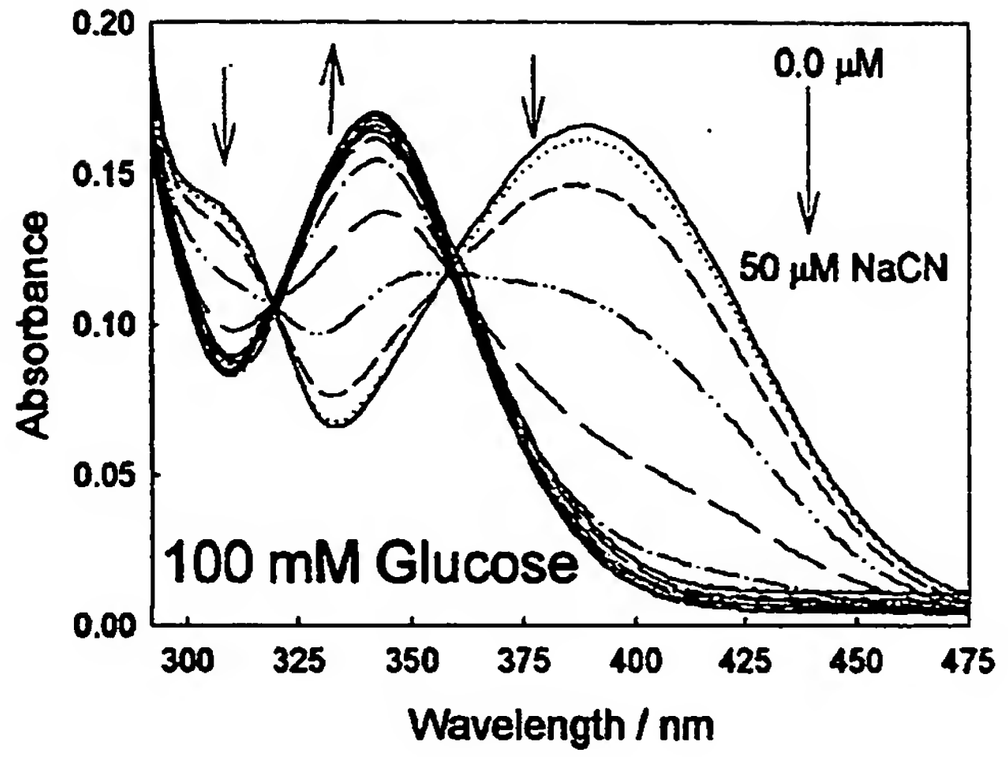

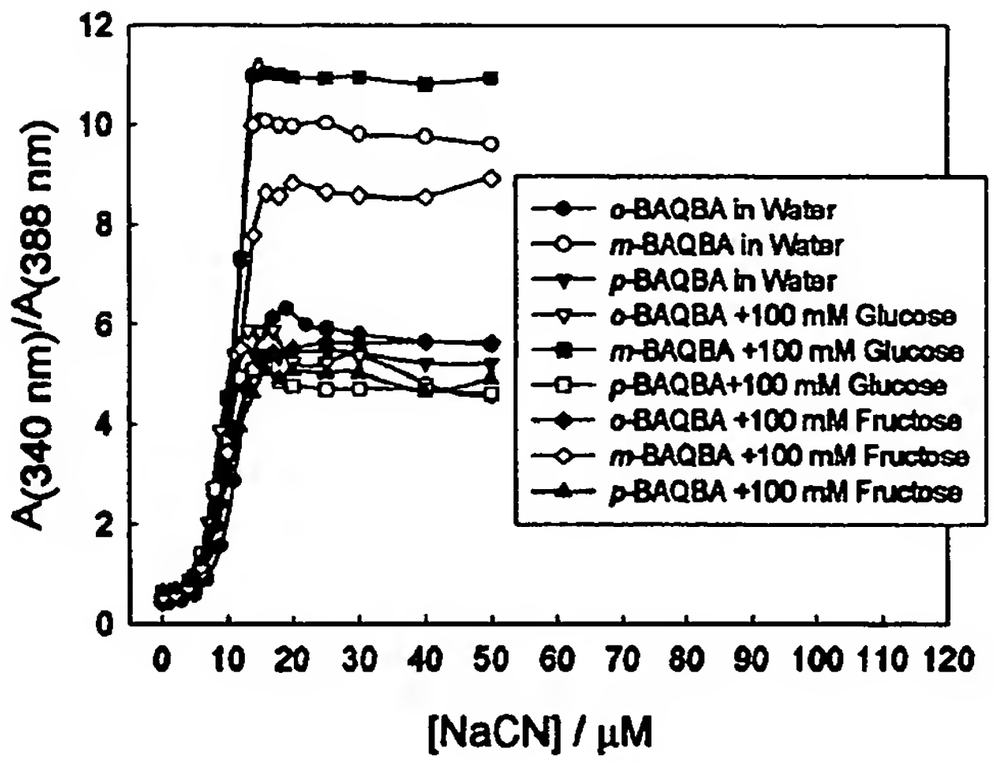

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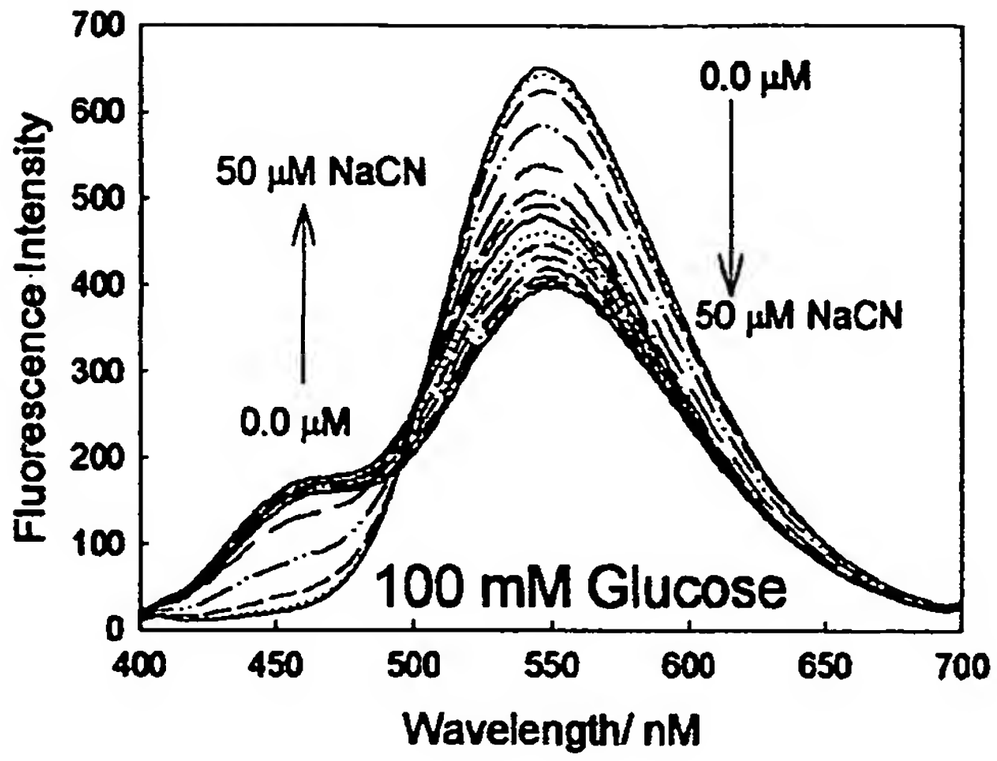

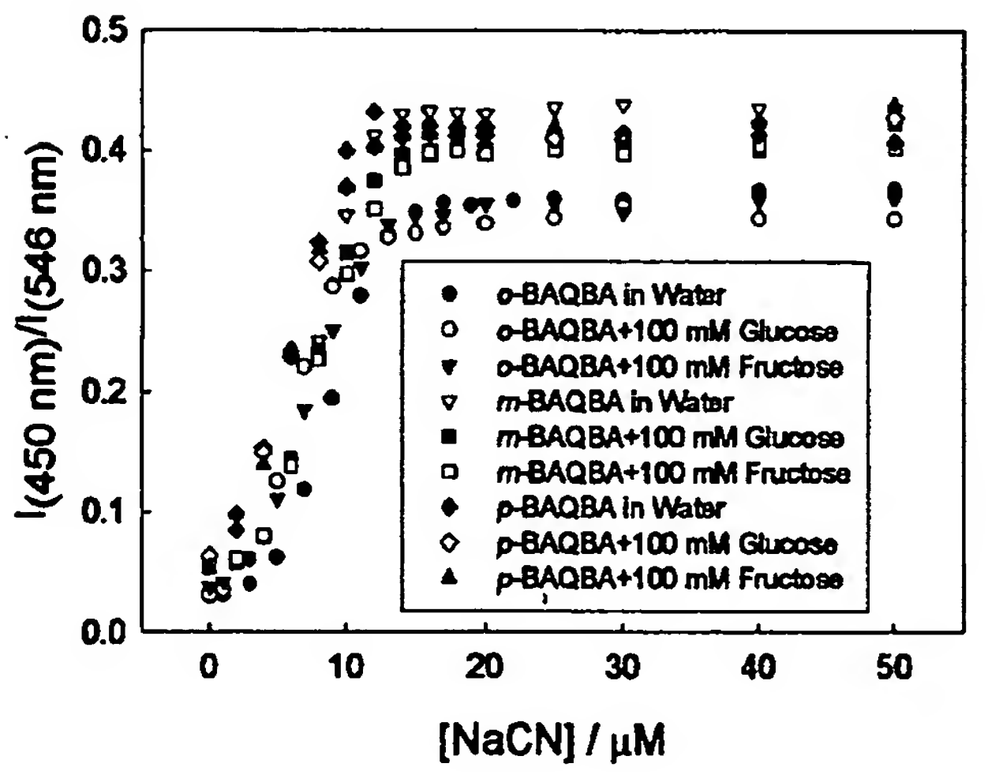

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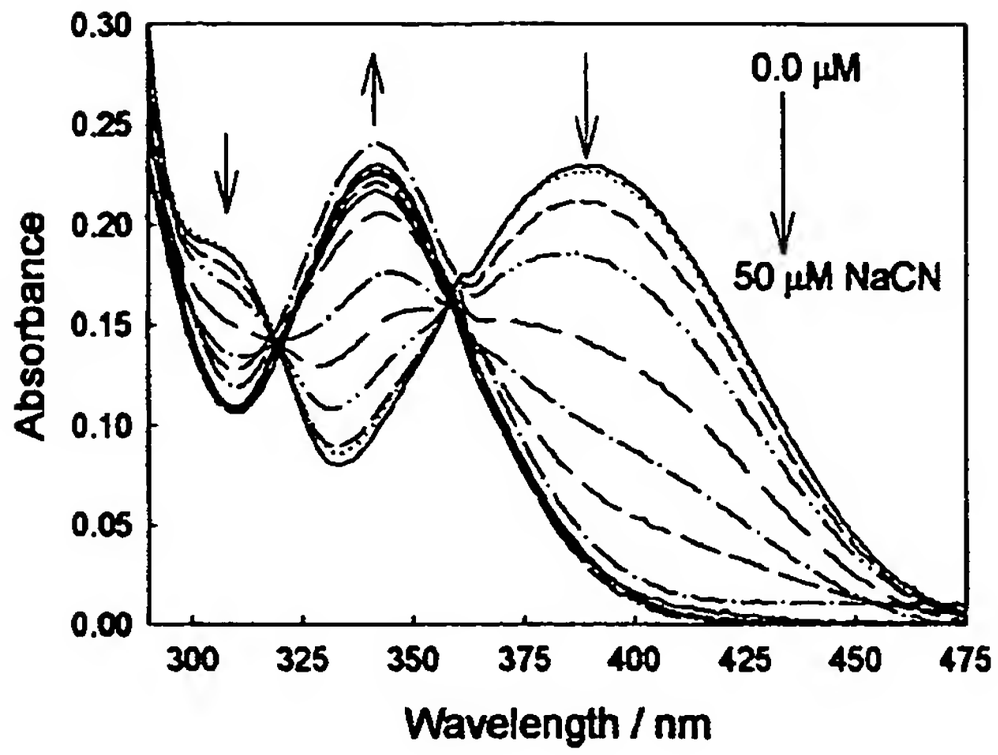

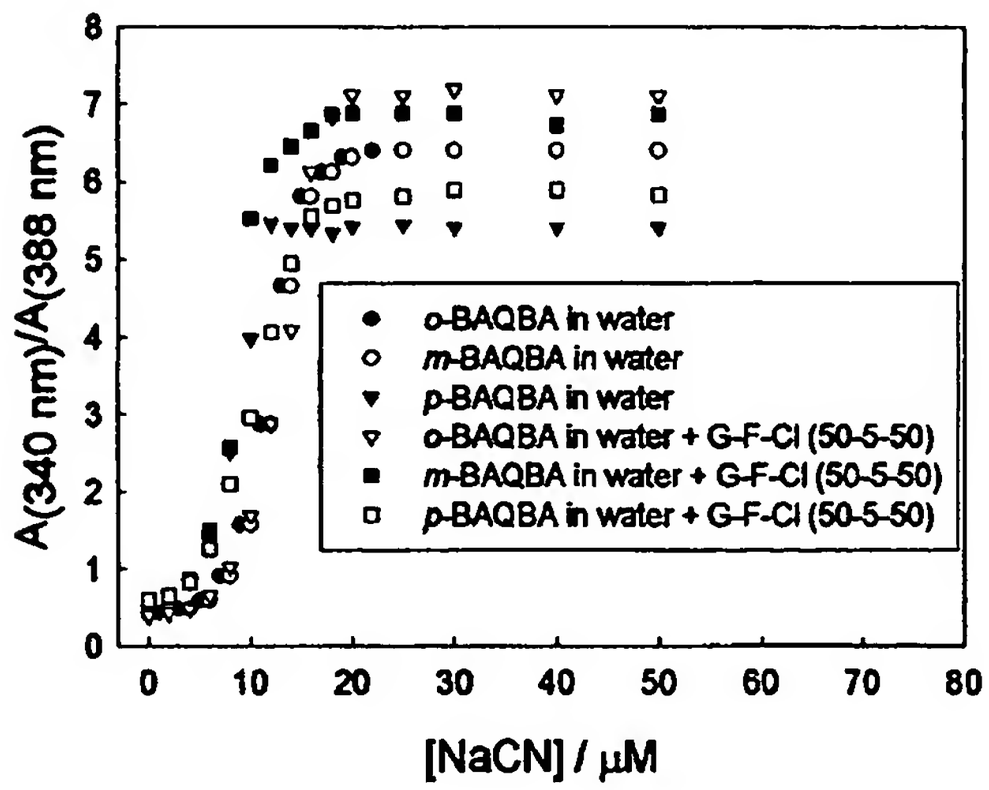

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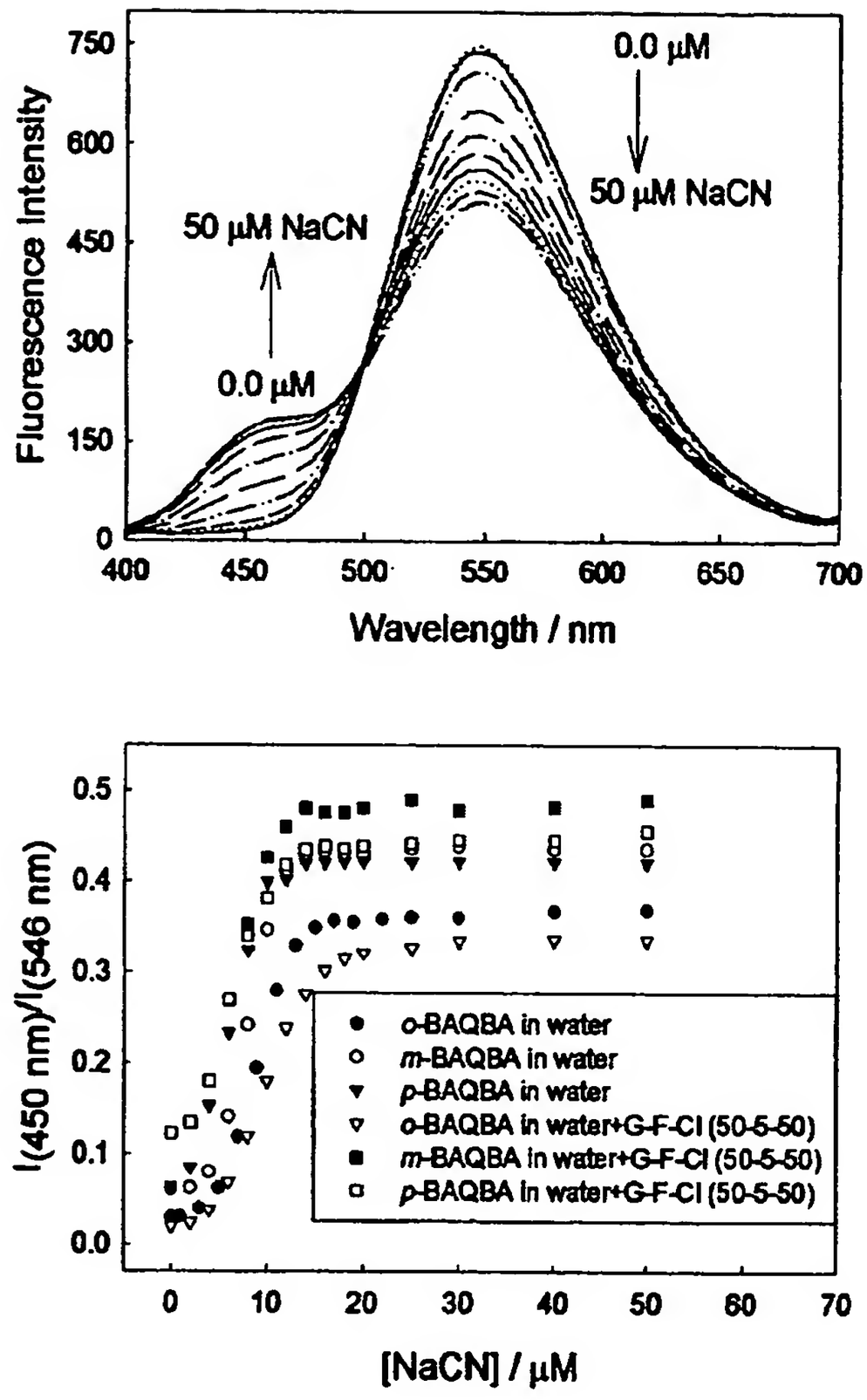

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Abstract

We characterize 3 new fluorescent probes that show both spectral shifts and intensity changes in the presence of aqueous cyanide, allowing for both excitation and fluorescence emission wavelength ratiometric and colorimetric sensing. The relatively high binding constants of the probes for cyanide, enables a distinct colorimetric change to be visually observed with as little as 10 μM cyanide.

The response of the new probes is based on the ability of the boronic acid group to interact with the CN^- anion, changing from the neutral form of the boronic acid group R-$B(OH)_2$ to the anionic R-$B(CN^-)_3$ form, which is an electron donating group. The presence of an electron deficient quaternary heterocyclic nitrogen center and a strong electron donating amino group in the 6-position on the quinolinium backbone, provides for the spectral changes observed upon CN^- complexation. We have determined the binding constants for the *ortho*, *meta* and *para* boronic acid probes to be 0.12, 0.17 and 0.14 μM^{-3}. In addition we have synthesized a control compound, which does not contain the boronic acid moiety, allowing for structural comparisons and a rationale for the sensing mechanism to be made.

Finally we show that the affinity for monosaccharides, such as glucose or fructose, is relatively low as compared to cyanide, enabling the potential detection of cyanide in physiologies up to lethal levels.

Keywords: Ratiometric cyanide probes, Colorimetric response, Boronic acid.

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/030066

International filing date: 16 September 2004 (16.09.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/503,689
Filing date: 17 September 2003 (17.09.2003)

Date of receipt at the International Bureau: 06 December 2004 (06.12.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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